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Abstract

More than 60 years ago, Geoffrey Harris described his “Neurohumoral theory” in which the regulation of pituitary hormone secretion was a “simple” hierarchal relationship, with the hypothalamus as the controller. In models based on this theory, the electrical activity of hypothalamic neurons determines the release of hypophysiotropic hormones into the portal circulation and the pituitary simply responds with secretion of a pulse of hormone into the bloodstream. The development of methodologies allowing monitoring of the activities of members of the hypothalamic-vascular-pituitary unit is increasingly allowing dissection of the mechanisms generating hypothalamic and pituitary pulses. These have revealed that whilst hypothalamic input is required, its role as a driver of pulsatile pituitary hormone secretion varies between pituitary axes. The organisation of pituitary cells has a key role in modifying their response to hypophysiotropic factors, which can lead to a memory of previous demand and enhanced function. Feedback can lead to oscillatory hormone output that is independent of pulses of hypohysiotropic factors and instead results from the temporal relationship between pituitary output and target organ response. Thus, the mechanisms underlying the generation of pulses can not be generalised and the circularity of feedforward and feedback interactions must be considered to understand both normal physiological function and pathology. We describe some examples of the clinical implications of the recognition of the importance of the pituitary and target organs in pulse generation and suggest avenues for future research in both the short and long-term.

Précis

Le Tissier and his colleagues revisit Harris's “Neurohumoral theory” to reassess the contribution of individual components of hypothalamic-pituitary-target organ axes in hormone pulse generation.

Introduction

Understanding the origin of anterior pituitary hormone pulses in health and how they are disturbed in disease is a long-standing question (1). The accepted "textbook" view has been that hypothalamic hormones are the dominant factors generating these pulses, based largely on the seminal experiments of Geoffrey Harris and colleagues that led him to develop the "Neurohormonal Theory" (2). Over sixty years later, the importance of hypothalamic factors is still unquestioned, however, it is apparent that their role in pituitary pulse generation is more complex than previously assumed. It is now clear that no single model system exists and that for each pituitary axis pulses of hormones are generated by a combination of hypothalamic input (3), pituitary response (4), short loop feedback (5) and target organ feedback (6). A clearer understanding of these interactions allows definition of their orchestration, essential for understanding the circuitries underlying physiology and behaviour (7).

In this review we will principally consider ultradian pulses of anterior pituitary hormones and divide their generation into two components: the regulatory inputs to the pituitary, both from the hypothalamus and peripheral organs; and the response of the pituitary gland. We will use specific examples to describe the processes and interactions involved and how their modification may lead to pathology. We will focus on the mechanisms underlying the generation of ultradian pulses. This review will not address the circadian pattern of pituitary hormone output: instead the reader is referred to three relevant articles/reviews (8-10).

Hypothalamic and target organ input in the generation of pituitary hormone pulses.

Hypophysiotropic neurons share features with many other neuronal cell types

The parvocellular hypothalamic neurons, which store and secrete hypophysiotropic hormones, have largely been considered as a separate class of neuron from those in other regions of the brain that have traditionally been classified by their small neurotransmitters. This view has now changed with studies of neurotransmitter contribution to the regulation of neurohormone release (11,12) and the realisation that many other neuronal circuits can be classified by their secretion of neuropeptides whether they have (e.g. somatostatin (SST) (13)) or do not have (e.g. kisspeptin (14) and orexin (15)) a neuroendocrine role. A unique feature of parvocellular neurons is that they lack post-synaptic targets, however, it has been shown that neuronal (16) and endothelial cell (17) inputs can modulate GnRH nerve terminal activity at the median eminence, similar to retrograde signalling at synaptic terminals. This highlights that the mechanisms and interactions which regulate hypothalamic parvocellular neurons should not be considered in any way distinct from those of other brain regions. A further recent realisation is that neurons considered as single populations may exist as multiple subtypes, which has been shown by single cell transcriptomics (18,19) and this is also true of hypophysiotropc neurons: regionally distinct gonadotrophin releasing hormone (GnRH) neurons have differential roles in GnRH pulse and surge generation (reviewed in (20)); and functional studies have identified two types of tuberoinfundibular dopamine (TIDA) neurons, only one of which is responsible for regulation of prolactin release (21). Further studies are likely to reveal heterogeneity in other hypophysiotropic neuronal populations with distinct roles in both pituitary regulation and modification of other hypothalamic functions.

Pulsatile hypothalamic output is not required for pulsatility in all pituitary axes

It is becoming increasingly clear that the previously accepted concept of the hypothalamus as the source of anterior pituitary hormone pulse generation is not applicable to all axes (Figure 1). This concept, of a simple hierarchal pulse generating relationship, is based on seminal studies in multiple species showing a concordance of the pattern of GnRH output and that of pituitary luteinising hormone (LH) and follicle stimulating hormone (FSH) (22-24). Afferent inputs to GnRH neurons are the origin of pulse generation (25), as demonstrated by a series of extensive and elegant studies of the GnRH system (20). As a consequence, the GnRH system has provided a paradigm for pulse generation in other pituitary axes, especially where the pattern of hypothalamic output can not be robustly measured. Identification of the factors regulating other axes show a further level of complexity, with multiple hypothalamic factors having synergistic (eg corticotrophin releasing hormone (CRH) and vasopressin (26)) or antagonistic (eg growth hormone releasing hormone (GHRH) and SST (27)) actions that affect the amplitude or duration of a pituitary hormone pulse but do not contradict the hierarchal relationship. However, recent studies in the adrenal axis has questioned the requirement for pulsatile hypothalamic input for a corresponding pulsatile pituitary output: constant CRH stimulation in conscious, freely moving rats resulted in pulsatile adrenocorticotrophic hormone (ACTH) and corticosterone release with a frequency unaltered from endogenous pulses (28). Similarly, in the thyroid axis constant infusion of thyrotrophin releasing hormone (TRH) in humans has been shown to result in pulses of TSH (29). This is not to say that TRH and CRH are not released into the portal circulation in pulses, where measurement has been made release is pulsatile (30-33) but this may be more related to maintaining responsiveness of target cells rather than pulse generation *per se* (34). Thus the paradigm established by the GnRH-gonadotrophin-sex hormone relationship (Figure 1 , left) may not hold for other axes, such as CRH-ACTH-cortisol (Figure 1, right), or indeed fully account for the relationship of GnRH and gonadotroph output at the time of the LH surge (20). Measurement of other hypophysiotropic factors with sufficient

temporal resolution to determine their relationship with pituitary hormone output or optogenetic manipulation of their hypothalamic neurons are required to determine this.

The electrical activity required for neurohormone release can be defined but is modified with physiological status

In those pituitary axes where pituitary hormones are released in pulses with a frequency of 10s-100s of minutes, it has not (to date) been possible to directly correlate the patterns of hypothalamic neuron electrical activity with their hypophysiotropic secretion. Calcium imaging and optogenetic manipulation to impose electrical activity with concurrent monitoring of pituitary hormone output (assumed to reflect hypothalamic factor release) have been used as alternative approaches to determine the minimal frequency and duration required to drive neurohormone secretion. This has been successfully applied to GnRH neurons, demonstrating that stimulation at 10 Hz (but not at frequencies below 5 Hz) are required for a duration of 2 minutes (but not 30 seconds) for generation of LH pulses (35), and to kisspeptin neurons, identifying them as a source of the GnRH neuron pulse generator (25,36). These studies assume that electrical activity and neurohormone release are correlated, which is likely in the short term, however studies of TIDA neuron electrical activity with simultaneous recording of dopamine output has demonstrated that this may not be true with changes in physiological status (37). In lactation, prolactin feedback no longer leads to dopamine release from TIDA neurons, which maintains the high level of the lactogen, but unexpectedly still leads to increased electrical activity. A similar disconnect between electrical activity and neurohormone release may occur in kisspeptin neurons which have an altered optogenetic stimulatory requirement in diestrus and ovariectiomized females (36) and a loss of neurohormone expression in lactation (38) .

Hypothalamic neuron coordination is required for pituitary regulation

Whatever the requirement for pulsatile hypothalamic output to generate pituitary hormonal pulses, there is an absolute requirement for hypophysiotropic regulation of the pituitary for

normal physiological function. This requires coordinated release from multiple neurons to ensure a sufficient concentration of neurohormone in the portal circulation to elicit a response from pituitary cells; for example, it has been shown that a minimum of 60 GnRH neurons are required for pulsatile LH release in mice (35,39) but five times that number are required for surge generation (40). More direct evidence for coordination has been shown by monitoring pairs of TIDA neurons, where the electrical activity of a proportion of cells are coordinated over a period of minutes (37). In both cases, there is an implication that a subset of the neuronal population is active at any one time, which may be important in avoiding fatigue. This provides a rationale for a large reserve population but also a requirement for interneuron coordination over both space and time; for example, a multi-layered spatial and temporal coordination of TIDA neurons remains stable over a period of days, which may underlie the sustained dopamine release required for inhibition of prolactin secretion (41). Such multi-layered organization of neuronal spiking frequencies are widely used for other brain-body functions, such as sleep, in both mammalian animal models and humans (42).

Intrinsic and extrinsic mechanisms coordinate hypothalamic neuron activity

The coordination of hypothalamic populations regulating pituitary secretion can occur through a number of mechanisms. In other brain systems, including those of other parvocellular neuronal systems, negative feedback and feedforward loops act as relatively simple networks to coordinate population activities (43) and there is evidence for a similar network-driven coordination of the hypophysiotropic neurons. These can be divided into intrinsic interactions within a population and extrinsic coordination requiring input from other neuronal cell types. Whilst there is evidence for both (as described below), a combination is likely to ensure the coordination required for robust pituitary regulation.

Intrinsic coordination

Coordination of the electrical activity of hypophystiotropic neurons has been reported, in particular for dopamine (37,44) and cultured GnRH neurons (45). Whilst this may suggest a role

for electrical coupling through gap junctions, these have been shown to be absent in both mouse TIDA (37) and GnRH (46) neurons. There may be species differences, however, since in rat TIDA neurons electrical coupling mediated by gap junctions has been described (44). An alternative mechanism underlying intrinsic coordination is chemical coupling and there is evidence for this regulating TIDA neurons via negative feedback loops, with TIDA neurons both releasing and responding to GABA (47). In addition, dopamine 2 receptor (D2R) at the TIDA neuron cell body mediates an ultrashort feedback loop leading to oscillatory activity in rats (48). A similar ultrashort autoregulatory loop has been described for GnRH neurons, which express GnRH receptors and have altered electrical activity in response to GnRH (49).

Extrinsic coordination

Input from the higher brain centres regulating hypophysiotropic neurons will obviously coordinate their activity, however, there is a clear role for intrahypothalamic regulation (50) and it is well recognised that SST and kisspeptin have important regulatory roles in GHRH (51) and GnRH output (14) respectively. Recent studies have determined specific roles for these extrinsic factors and defined key steps in their regulation of neurohormone output. The inhibitory action of SST has been shown to counterintuitively lead to stimulation of GHRH neurons as a result of an initial fast and transient direct inhibition of the GHRH neuron itself, followed by a delayed inhibition of both excitatory glutamate and inhibitory GABA inputs (52). Optogenetic manipulation has identified kisspeptin as the GnRH pulse generator (25) and other studies have shown that firing of kisspeptin neurons is modulated by steroid feedback (53). Thus, feedforward loops are key features of both of these hypophysiotropic systems. A further complexity in the extrinsic inputs regulating hypophysiotropic ouput may be their subcellular location. Kisspeptin has been shown to have differential effects at the GnRH cell body compared with the nerve terminals at the median eminence (16), where a role for local endothelial nitric oxide production has been suggested as a local synchronising signal (54).

The median eminence plays a role in coordinating and modifying hypothalamic output

The final step in the output of hypophysiotropic hormones is their release at the median eminence (ME). Release from a large number of neurons into this richly vascularised structure, with convoluted loops collecting output from a large release area, optimises both the amplitude and duration of neurohormone pulses in the portal circulation, whilst avoiding neuronal fatigue and exhaustion (41). In addition to roles in the coordination of hypophysiotropic factor release (see above), the ME may actively modify output by alteration of access of nerve terminals to the rich capillary bed by either changes in localisation, which has been shown to vary with age for GHRH neurons (55), or tanycyte ensheathment, shown for GnRH neurons to vary at different stages of the oestrous cycle (56).

Peripheral inputs can generate pituitary hormone pulses

The importance of target organ feedback in the regulation of hypothalamic-pituitary axes is well recognised and incontrovertible, balancing the feedforward regulation by hypothalamic and pituitary factors. An excellent example of this is the differential regulation of LH and FSH by ovarian inputs, with reduced inhibin and increased progesterone feedback actions on the pituitary generating a second phase of FSH (but not LH) at proestrus and estrus (reviewed in (22)) (Figure. 1, left panel).

Remarkably, recent studies inspired by mathematical modelling have shown that target organ feedback itself can act as a pituitary hormone pulse generator, as the fast feedforward action of ACTH on the adrenal gland and delayed feedback of glucocorticoids can generate pulses of both hormones with invariant CRH (reviewed in(6)) (Figure 1, right panel). Since an intra-adrenal glucocorticoid feedback loop has recently been suggested from modelling (57), this suggests that the adrenal gland itself may be the primary pulse generator in the hypothalamic-pituitary-adrenal axis in the absence of stress. The extent to which similar temporal relationships of hypothalamic-pituitary regulation and feedback exist for other axes is currently unclear, although a delayed feedback of prolactin on dopamine neurons (41) may have an important

impact on dopamine tone, facilitate increased secretion of prolactin and lead to the reported ultradian pulses of basal prolactin secretion (58).

The potential interactions whereby feedback can generate or modulate pulsatile pituitary hormone secretion are complex and may include:

- the temporal relationship between the feedforward and feedback regulation, which is complicated by the feedback occurring at multiple levels; for example, the differential feedback actions of ovarian steroids are mediated by rapid non-genomic and classical steroid receptor actions in both the hypothalamus and pituitary during the oestrous cycle, with effects that are dependent on receptor isoform expression and downstream signalling (reviewed in (22,24)).
- the sensitivity of the system to feedback, exemplified in the thyroid axis, where differential expression of thyroid hormone receptor beta isoforms results in its relative increase in sensitivity to thyroid hormones (59), providing an anticipatory mechanism to protect peripheral organs from overexposure to these hormones (34).
- differential feedback at the level of the hypothalamus and pituitary. Again the thyroid axis provides an excellent example of this, since feedback to the hypothalamus is dependent on active transport of thyroid hormone at the level of the median eminence (60) but is enhanced by post-translational modification of type 2 deiodinase (61).
- multiple factors feeding back on a single cell type; for example, dopamine neurons will be exposed to GABA, dopamine and prolactin feedback, with different time scales (41,47,48).

Thus, it is possible that feedback occurs at both pituitary and hypothalamic levels and at multiple sites within each organ, through the action of multiple factors on a single cell type, or both. This is further complicated when consideration of feedback to higher brain centres is included; for example, glucocorticoid feedback on the limbic system and brain stem (62).

Intrapituitary regulation of hormone pulse generation

It is perfectly feasible that the anterior pituitary gland would simply passively respond to hypothalamic and peripheral inputs, with its cells simply acting as an amplifier of hypothalamic regulation that is modulated by feedback from target organs. However, it is now apparent that this is not the case, with an active role for the pituitary mediated by the structural organisation of its component cell types affecting how they receive, interpret and translate hypothalamic and peripheral signals into highly ordered hormone pulses. This was previously suggested by a disconnect in the output of dispersed pituitary cells compared with those in the intact gland (63) but is being increasingly demonstrated and dissected using a combination of mouse models and technological innovation which allows both temporal and structural imaging (64,65).

Pituitary cells are organised as intermingled homotypic cell networks.

Large-scale 3D imaging of genetically-modified mouse models expressing fluorescent proteins under hormone promoter control has revealed the structural and functional organisation of the pituitary gland and its rich vascularisation (4). This has described the developmental program of the topological organisation of differentiated cells throughout the gland (63,66) from early fetal life to adulthood, and has demonstrated a role for early-differentiated cells (e.g. corticotrophs) in controlling both the positioning and expression of late-differentiated cells (e.g. gonadotrophs) (67) . Contact between homotypic cells and organisation of characteristic morphological features occurs soon after endocrine cell differentiation, before the onset of hormone secretion and leads to cell network formation (67,68). Among the signal molecules involved in cell network architecture and plasticity, detailed analysis of the cadherin family has revealed a 'bar-coding' expression of cadherins within distinct pituitary cell populations from both mouse models (69) and humans (70), which has also been proposed as a marker rule for discriminating invasiveness in GH and PRL adenomas.

Pituitary cell networks share fundamental properties with other biological networks including metabolic signalling networks in yeast and bacteria (71), where a prevalent feature is that of

simple assembles of elements (so-called network motifs) which recur within the population (72). The organisation of the various pituitary cell types into distinct motifs suggests that there will be differences and similarities in their role in axes function. The spatial organisation, and its plasticity throughout life, is exemplified by the GH and prolactin cell networks. Upon sexual maturation there is a transient increase in the generation of multiple clusters of contacting GH cells (illustrated in Figure 2) in males but not females in the wings of the pituitary, coincident with an increase in the highly ordered GH pulses which control liver insulin-like growth factor 1 production (64,68). The importance of these GH cell clusters as network motifs which lead to increased body growth is suggested by the correlation of their formation with growth rate (68) and the finding that GH-deficient animals are normal in size if the GH cell clusters are preserved (73). In contrast, PRL cells are organised as multiple honeycomb network motifs (like an orange peel) which are more prominent in lactating females and display experience-dependant plasticity as they remain after weaning (74). This altered network organisation has been shown to result in enhanced prolactin output in subsequent lactations (74) and may have a role in reducing the tonic output of prolactin in reproductively experienced rats (75) through an enhanced response to dopamine inhibition (76).

The vasculature has a role in signal input to pituitary cell networks

Networks of endocrine cells do not work alone but form a functional continuum with other elements within the pituitary gland, including the vasculature. Network motifs and the rich plexus of fenestrated capillaries are topologically organized in a manner which is distinct for each endocrine cell type (Figure 2), and may therefore reflect their different secretory temporal dynamics (63). Initial cell network formation begins before the first capillaries invade embryonic pituitary tissue (67) and loss of the pituitary cell transcription factor Prop1 leads to a failure of organ vascularization (77). Thus, endocrine cell networks have a stimulatory and organisational role in patterning capillary invasion.

The organisation of the vasculature with pituitary endocrine networks may have a significant impact on the amplitude and timing of exposure of pituitary cells to hypothalamic regulatory factors since seminal studies of the portal vasculature have shown that hypophysiotropic nerve terminals specifically abut portal vessels which irrigate specific pituitary regions (78,79). In addition, there is a highly dynamic regulation of the distribution of incoming secretagogues within the pituitary through altered blood flow dynamics within the capillary bed of the pituitary (80) but rapid transit of signalling molecules (in a range of seconds) throughout portal fenestrated capillaries (81). This suggests differential timing of exposure of different regions of the pituitary to hypophysiotropic factors, resulting in a complex dynamic of sequential stimulation, with "scout cells" stimulated before other cells within a homotypic network. Since networks have a functionally coordinating role (see below), this pattern of exposure may lead to synergistic interactions and potential role(s) for specific subsets of cells ensuring robust responses to stimulation. The contribution of the blood system to pituitary hormone pulsatility also involves the fate of hormones from their releasing site towards the bloodstream, which will ultimately deliver the appropriate pattern of hormone pulses to the peripheral target, as well as coordination of oxygen and nutrient supply with the metabolically demanding processes of (80).

Pituitary networks coordinate response to regulation

A functional role for homotypic pituitary cell networks in determining endocrine output is suggested by their formation before the onset of hormone secretion and stimulation by secretagogues and functional reorganization in response to altered demand (68,74). This has been confirmed by *ex vivo* analysis of calcium and gene expression dynamics in homotypic cell networks, with coordinated responses to stimulation that are severely dysregulated when networks are disrupted (64,73,82,83). Gap junction coupling contributes to this network coordination (64,74,82), however, this does not preclude roles for paracrine factors between both homotypic and heterotypic cells (reviewed extensively in (84)), such as secreted TSH which exerts an ultrashort negative feedback which could drive ultradian TSH pulses (34). It is

also possible that pituitary networks mediate predictive programming, or priming, of axis function since in prolactin cells, the increased organisation associated with lactation persists for months after weaning and leads to enhanced function (74). Similarly, the increased clustering of somatotrophs at puberty could be considered as a priming event for increased GH release, although in this case the effect is transient (64). It is possible that similar transient changes in organisation enhance the altered sensitivity and self-priming of gonadotrophs to GnRH stimulation (85) since increased cell movement and number of cell processes have been described in this cell type in response to GnRH (86) and estradiol (87). Thus both structural and functional organisation of pituitary endocrine cells as intermingled 3D cell networks have important roles in the amplitude and dynamics of hormone secretion which can be modified throughout life.

Pituitary cells are heterogeneous

Whilst pituitary networks mediate a coordination of cell activity, individual cells also show functional heterogeneity, which may reflect transient or permanent differences in cell activity. This is exemplified by the identification of a small subset of prolactin cells which act as pace-makers, or network nodes, synchronizing the activity of nearby homotypic network cells (65). It is these pace-making cells which mediate the altered function of prolactin cells between first and second lactation, showing an ability to store a cellular memory of previous demand that also leads to an enhanced output when rechallenged (akin to learning). Over a timescale which is an order of magnitude longer than that of secretory activity, prolactin gene expression in lactotrophs has also been shown to be heterogeneous (82). A continuous distribution of both transcription rates and switches were found in this study, although interestingly this was locally spatially coordinated by the prolactin cell network, suggesting that the mechanisms underlying homotypic cell coordination can act over a wide range of timescales. Similar functional heterogeneity has been described for other pituitary cell types, which can result in stereotypic variable responses to stimulation which have previously been considered to be stochastic (88).

Further studies are required to determine if these heterogeneous responses identify a distinct sub-population of pituitary cells or transient activity states, which will likely be identified by high throughput sequencing technologies and/or photolabeling of individual cells in situ (89).

Pituitary cell secretion is integrated to shape pulsatile circulating hormone

The rate of entry of a secreted pulse of pituitary hormone to the bloodstream and exit from the gland will be determined by the relationship of cell networks with the pituitary microvasculature, where perivascular spaces act as gate-keepers for hormone transfer to the capillary lumen (80). Once in the systemic circulation, a pulse of hormone will be combined with that released in previous secretory events, resulting in the concentration of circulating hormone is an integration of basal and pulsatile release which is dependent on hormone half-life (1,4). Since the half-life of pituitary hormones can be modified by circulating binding proteins (90) and post-translational modification (34), both of which also modify their bioavailability, the pattern of exposure of a receptor to a hormone pulse is complex and will not simply mirror that of pituitary release.

Implications for health and disease

A recognition that pulses of pituitary hormone are generated and modified at multiple levels has important implications for the study of normal axes function but, importantly, also for how dysregulation occurs and for identification of therapeutic targets. This is particularly relevant to pituitary tumours, where hormone output is largely independent of hypothalamic stimulation. In Cushing's disease, for example, there is a marked increase in basal secretion of both ACTH and cortisol and pulsatility is preserved (91) but becomes less ordered (92). Significantly when considering the interaction between glands, there is a decrease in the potency of cortisol stimulation by ACTH (93) and a reduction in the pulse correlation of the two hormones (91). Similar changes in the orderliness of pituitary hormones has been described for other types of

pituitary adenoma, which importantly is largely normalised by surgical but not medical treatment (94).

It is possible that the interactions between the hypothalamus, pituitary and target organs in generating pulses may have a significant role in a number of endocrine disorders, and thus should be considered as a potential mechanism leading to disease as well as new targets for therapy. For example, multiple studies to identify defects leading to polycystic ovarian syndrome (PCOS) have focused on dysregulation at the levels of the hypothalamus (95,96) and ovary (97). Aspects of the disorder, such as the potential role of hyperinsulinemia in loss of fertility have been studied at the level of the pituitary (eg (98)) but overall there has been a paucity of studies of the role of the pituitary. It is possible that increased LH pulse amplitude (but not pulse frequency) found in PCOS patients may be a result of an alteration of pituitary function and further research into a potential role of the pituitary in this disorder is warranted. Given that PCOS is a heterogeneous syndrome, it is possible that there are multiple aetiologies that involve all levels of the HPG axis some of which may be secondary to the primary defect but nevertheless require improved understanding and may be targets for therapy.

The multi-level regulation of pulsatile hormone output also has important implications for diagnosis of dysfunction. An excellent example of this is provided by the thyroid axis, where the "normal" concentration of circulating TSH can vary between individuals (34) and may be altered for prolonged periods following normalisation of axis function following hyper- or hypothyroidism, referred to as hysteresis (99). This may occur as a result of differential rates of feedback regulation at different levels of the axis; for example, a reduction in hypothalamic TRH gene expression in response to normalisation of hypothyroidism (100) would be expected to be rapid in comparison with any change in pituitary thyrotroph cell mass (101), resulting in an alteration of the set point for each level of the axis.

Future research/perspective

Models of pituitary pulse generation:

It is clear that there has recently been substantial progress in understanding the contribution of individual components of hypothalamic-pituitary-target organ axes to pulse generation. However, the challenge is to dissect the temporal interactions of these individual components, which requires *in vivo* studies with simultaneous monitoring of system inputs and/or outputs. The use of imaging technology to observe cell activity has been instrumental in much of the recent progress, since it allows both temporal and spatial resolution of activities. Further development of *in vivo* imaging technology, such as the use of gradient index lenses (102), is required to facilitate this. An important consideration in these studies is the silencing of multiple hypothalamic neuron populations by anaesthesia (80), meaning that imaging in awake, freely moving animals is required. Furthermore, monitoring of cell stimulation and activity currently relies on imaging of specific cell signals, such as calcium as a surrogate for monitoring both inputs and outputs (eg (64)). The development of methodologies to specifically monitor receptor activation, such as "sniffer cells" (103) or luciferase monitoring of G-protein coupled (104) and cytokine receptor (105) activation may allow more direct measurement of both stimulatory inputs and hormonal output.

The heterogeneity of both hypothalamic and pituitary cell populations in generating hormone pulses has been a notable feature throughout this review. Whilst it is possible that this may represent stochastic cell activity in some cases, in others it has been found to be deterministic (88). Since multiple studies have demonstrated that only a small proportion of hypothalamic (eg GnRH (40)) or pituitary (eg GH (73)) cells are required for apparent normal function, the question remains whether the heterogeneous responses reflect sub-populations with specific physiological functions. Identifying the differences in protein expression and post-translational modification that may underlie heterogeneity may be suggested by single cell transcriptomics of cells with specific activities. These studies will not define whether the heterogeneity reflects transient activity or specific sub-population of cells, however they will suggest factors that define sub-populations of cells currently primarily defined by the hormone they produce. The use of cell tracing methodologies, optogenetics and Designer Receptors Exclusively Activated by Designer Drugs (DREADDS), which have already

made dramatic contributions to understanding of hypothalamic-pituitary axis function (eg (25,106)), will allow confirmation of sub-population identity and study of their function. This will be facilitated by the use of CRISPR/Cas technology in combination with adeno-associated viral delivery of factors to manipulate cell function (107). Such single cell transcriptomic approaches have been successfully applied in other systems, with consequences for identifying novel therapeutic targets (108) and for sub-population identification in the brain (109).

Our identification of features akin to memory and learning in the pituitary suggests a potential role of the gland in physiological programming. For example, a persistent alteration in corticotroph activity has been described in adult sheep exposed to a brief period of maternal perinatal undernutrition (110). There is a clear requirement for further investigation of epigenetic alteration of gene expression in such models, however, persistent changes in pituitary cell organisation leading to altered network functions are also possible. This will require *in vivo* analysis, as well as a clearer understanding of the mechanisms underlying network-mediated regulation of axis function. The single cell transcriptomics and cell manipulations described above may allow identification of potential mechanisms underlying network function. Mathematical modelling and tissue engineering may aid understanding of how different network motifs affect cell-cell coordination.

Finally, the role of the vasculature in modifying temporal and spatial regulation of pituitary function and clearance of secreted hormone from the gland is an area that requires further analysis which should be possible with optogenetic manipulation or the use of DREADDS. These may also be used to determine how the relationship of pituitary cell networks and the vasculature is altered in adenoma formation (111), as well as in other axes dysfunctions such as PCOS.

Translation to the clinic:

The mechanisms leading to pituitary hormone pulse generation that are currently being elucidated in rodent models are likely to generally translate to those in humans, however, there are clear species differences in the physiology of pituitary axes (eg prolactin (112)). Analysis of post-mortem pituitary tissue will allow comparison of network organisation and their relationship with the vasculature, as

well as the expression patterns of factors identified as intermediates in network function. It is also possible that fresh post-mortem tissue and adenomas from patients will allow some functional analysis of human pituitary function and correlation with that of rodents. The analysis of organoids of pituitary tissue differentiated from induced pluripotent stem cells (113) will most likely establish whether mechanisms underlying rodent network function are recapitulated in humans, as well as the consequences of mutations identified in patients presenting at clinic with pituitary dysfunction.

Many of the current protocols for diagnosis of pituitary dysfunction may not fully interrogate the complex interactions leading to pulse generation, which may explain why, for example, current provocation tests misdiagnose GH axis function in a proportion of patients (114). Rodent models will allow the development of tests which can more fully define hypothalamic and pituitary functionality and determination of parameters that are affected when physiology is altered; for example, at puberty and in obesity. It is also possible that an *in vivo* assessment of pituitary function may be possible through an improved understanding of how pituitary blood flow relates to function, as this may be assessed in patients through, for example, ultra-fast ultrasound imaging (115).

Identification of pituitary cell networks may also affect whether and how stem cell therapy could be used for treatment of hypopituitarism, which would be simplified in humans through transphenoidal access to the pituitary. Whilst there has been substantial progress in identifying pituitary stem cells (116,117) and developing protocols for differentiation of embryonic and pluripotent stem cells to pituitary tissue (118,119), it is currently unclear whether stem cells would be capable of self-organisation or integration into existing pituitary cell networks. This is further complicated by the identification of functional heterogeneity and programming of cell function by previous demand. A naïve stem cell may be capable of differentiation to a lactotroph and integration into an existing network, for example, but may not be functionally equivalent to a cell exposed to the demands of lactation. Furthermore, the prevalence of pituitary adenomas with aberrant network function reflected in disorganised pulsatile output (94) suggests that a failure to fully integrate or recapitulate normal network function may be a risk for the development of pathology. Injection of lineage traced stem

cells into the pituitaries of rodent models of hypopituitarism and functional imaging of their function may establish the potential for stem cell therapy in humans.

Conclusion

The elegant and ground-breaking experiments of Harris and colleagues were prescient in their use of *in vivo* models which allow multi-organ interactions. It is now clear that in such an interactive system the concept of a hierarchy is not appropriate except in the identification of a pulse generator, which in the case of the HPA axis, at least, may not be the hypothalamus. This does not suggest that the mechanisms and principles underlying the relationship of the hypothalamus, pituitary and target organs differ between axes but the strength and timing of inputs lead to unique features. Thus, the concepts underlying Harris's Neurohormone Theory of regulation of pituitary axes have borne the test of time but new levels of complexity have emerged that require consideration of interactions between multiple components of the axes.

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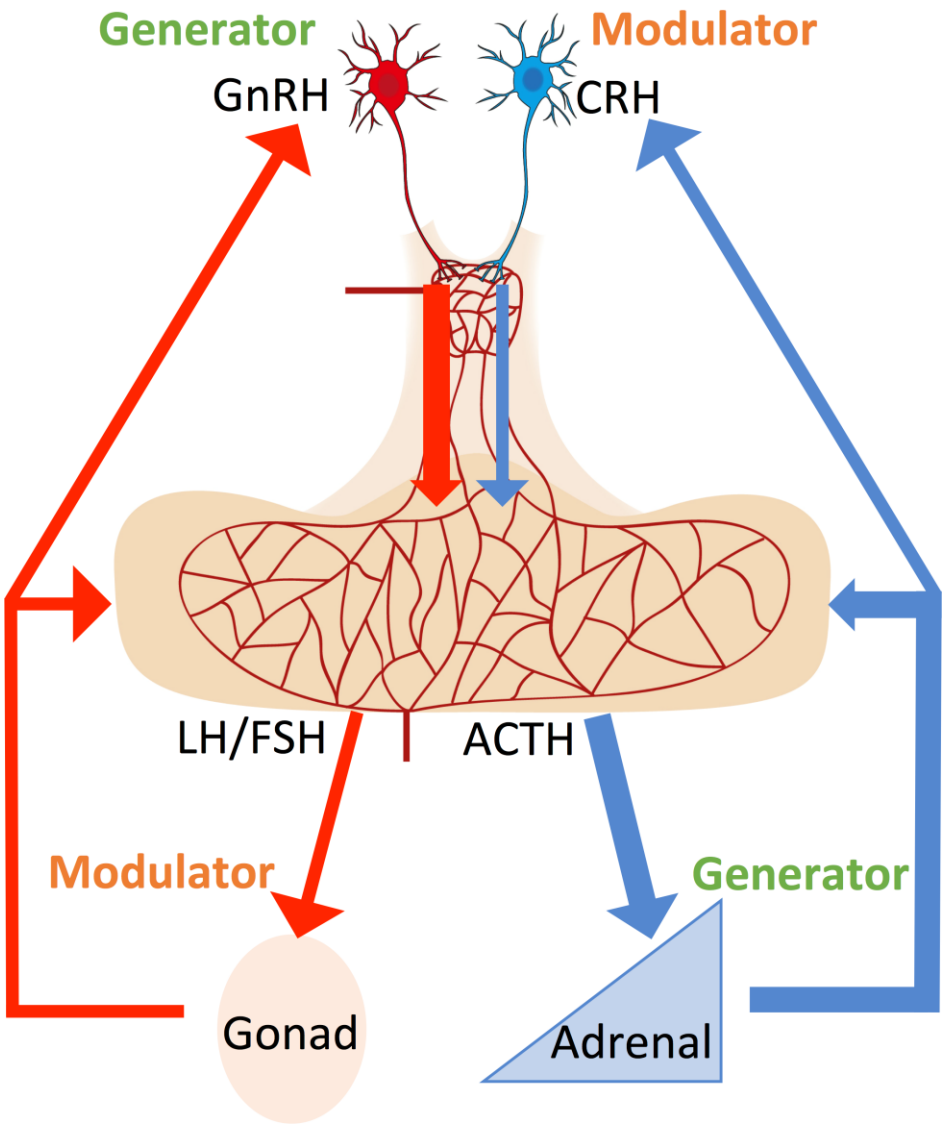
Figure legends:

Figure 1. A simplified schematic showing the contrasting regulation of pituitary hormone pulse generation between the hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-adrenal (HPA) axes. In the HPG axis, the pulse generator is localised in the hypothalamus, where afferent inputs from kisspeptin neurons (25) and neuronal feedforward loops (53) lead to pulsatile release of gonadotrophin releasing hormone (GnRH). This results on the release of pulses of luteinizing (LH) and follicle stimulating (FSH) hormone, stimulating secretion of steroids from the gonads which feedback on a relatively slow timescale to both the pituitary and hypothalamus. In contrast, in the HPA axis the rapid actions of adrenocorticotrophic hormone on the adrenal gland and delayed feedback of glucocorticoids on the anterior pituitary is the source of pulse generation, with corticotrophin releasing hormone (CRH) having a modulatory role (28).

Figure 2. The growth hormone (GH) cell network and its relationship with the vasculature. Two-photon imaging of the pituitary of a GH-GFP transgenic mouse with capillaries labelled with gelatine-rhodamine (red). GH cells are organised into a homotypic topologically organised network of cell clusters which are linked by strings of single cells. The cell network is closely associated with capillaries, which are aligned with strings of cells and surround the clusters.


Generator
Modulator
GnRH
CRH
LH/FSH
ACTH
Modulator
Generator
Gonad
Adrenal